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Evidence for the Existence of Distinct Nitroreductases in 'Salmonella typhimurium' Roles in Mutagenesis

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16. ABSTRACT

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JOURNAL ARTICLE

Evidence for the existence of distinct nitroreductases in *Salmonella typhimurium:* roles in mutagenesis

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Abstract

Previous studies on the mutagenicity of nitroheterocyclics and nitrated polycyclic aromatic hydrocarbons had indicted the probable existence in *Salmonella typhimurium* of a multiplicity of nitroreductase activities of varying specificities which are required for the expression of the mutagenicity of nitro-containing chemicals. In the present study evidence is presented that these activities reside in different gene products: (a) strains totally lacking in the nitroreductase which recognizes niridazole and related substances are not mutagenized by this group of chemicals and yet they are fully responsive to the mutagenic action of dinitropyrenes; (b) double mutants which have lost both types of specificities can be constructed. Finally, the presence of a third type of nitroreductase with a specificity for 4-nitroquinoline-1-oxide is implied by the finding that the nitroreductase-deficient strains described herein retain full sensitivity to the mutagenic action of this chemical.

Introduction

Nitro-containing polycyclic aromatic hydrocarbons (NO_2-PAH's)* are being recognized as ubiquitous products of mobile as well as stationary combustion processes and as such are present in the environment (for literature citations see ref. 1). The mutagenicity of many of these NO_2-PAH's for bacteria is elevated (2 – 5) and this may be cause for concern. In order to determine whether the high genetic activity of these chemicals for bacteria is the result of metabolism unique to microbes (6), we have investigated the mechanism whereby these chemicals are reduced enzymatically by the bacteria to the corresponding hydroxylamines, presumably the penultimate mutagens. These studies suggested that bacteria contain several classes of nitroreductases (NRs) which recognize different substrates (7). Thus NR-deficient derivatives of *Salmonella* TA98 isolated as nitrofuran-resistant are not mutagenized by niridazole (NIR), nitronaphthalenes and nitrofluorenes but are fully sensitive to several nitropyrenes (1,2, 8 – 12), suggesting that this "classical" NR does not reduce nitropyrenes to penultimate mutagens. In view of the fact that it had been established that the mutagenicity of nitropyrenes was due to covalent binding of metabolites to cellular DNA (1,9,13), we investigated the possibility that sensitive bacteria possessed NRs other than the "classical" one which acted upon nitropyrenes. This led to the isolation of a bacterial strain which was not mutagenized by 1,8-dinitropyrene (1,8-DNP), was also resistant to mutagenesis by 1,3-dinitropyrene, was intermediately sensitive to 1,6-dinitropyrene, and was sensitive to trinitro- and tetranitropyrenes and to NIR (7,12). These observations suggested the possible existence of a multiplicity of NRs with different specificities. Moreover both the strains lacking the "classical" NR which recognizes nitrofurans and the one deficient in the enzyme reducing dinitropyrene are fully sensitive to the mutagenic action of 4-nitroquinoline-1-oxide (NQO) and a nitroacridine (7,14), this indicated the possible existence of additional NRs.

These findings suggest that major studies would have to be undertaken to determine (a) the total number of NRs; (b) the location and function of the genes regulating their synthesis; (c) their physical chemical characteristics; (d) their substrate specificities, and (e) finally whether such enzymes are also present in eukaryotes. However before embarking upon such extensive studies we wished to eliminate the possibility that we were dealing with a single enzyme with altered substrate specificities as a result of mutationally introduced changes in the amino acid sequence of a single gene product.

We used two approaches to investigate this possibility (a) by determining whether double mutants, lacking both NRs (i.e. the "classical"' one and the one which reduces 1,8-DNP to a mutagen) were capable of existing, and (b) whether mutants totally lacking the "classical" NR, even an inactive gene product, were sensitive to mutagenesis by dinitropyrenes. Our findings indeed indicate that *Salmonella* contain at least two separate classes of NRs: one which recognizes nitrofurans and the other dinitropyrenes.

Materials and Methods

Salmonella typhimurium TA98 was obtained from Dr B.N.Ames (15). Derivatives lacking the "classical" NR (TA98NR) were isolated as NIR-resistant by plating overnight cultures on Columbia agar plates containing NIR (5 μg/ml). Similarly strains lacking the enzyme capable of reducing 1,8-DNP (TA98/1,8-DNP) were isolated as mutants capable of growing in the presence of 50 μg of this chemical per ml. Resistant colonies were recloned and the retention of the deep-rough character was determined by sensitivity to crystal violet while the presence of the plasmid (pKM101) characteristic of TA98 was ascertained by ampicillin-resistance (15). All selected mutant clones were then tested for mutagenic response to a panel of mutagens, including non-nitro-containing chemicals. Only mutants retaining full sensitivty to the mutagenicity of non-nitro-containing chemicals were used for further studies (7,16).

Double mutants were selected by repeating the above procedure, i.e., TA98NR were rendered resistant to 1,8-DNP and conversely TA98/1,8-DNP, the 1,8-DNP-resistant strain of TA98 (7) was made resistant to NIR.

The isolation of mutants completely devoid of the "classical" NR using a frameshift mutagen (9-aminoacridine) and NIR as the selecting agent has been described (17).

The mutagenicity assay was carried out as described previously (15). No microsomal activation mixtures were used. Every concentration was tested in triplicate on at least two separate occasions. Specific mutagenic activities were calculated from the linear portions of the dose-response curves.

1,8-DNP (at least 99% pure) was prepared as described previously (9); NQO was obtained from the National Cancer Institute Chemical Repository and NIR from Ciba-Geigy.

*Abbreviations: NO_2-PAH's, nitro-containing polycyclic aromatic hydrocarbons; NR, nitroreductase; NIR, niridazole; 1,8-DNP, 1,8-dinitropyrene; NQO, 4-nitroquinoline 1-oxide.

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Results and Discussion

The original strains lacking the "classical" NR (e.g., TA98NR) were isolated as derivatives of TA98 resistant to nitrofuran or to NIR (8,16). However, because the selecting agents were also mutagens primarily of the base-substitution type, it is very probable that the NR-deficient strains resulted from base-substitution mutations. Thus, they can be expected to contain modified enzymes (i.e., amino acid substitution) that are either inactive, or of greatly reduced enzyme activity, or of a different substrate specificity, such that they acquired a specificity for nitroarenes. However, it is to be expected that strains rendered deficient in "classical" NR as a result of a frameshift mutation would be completely devoid of enzyme, even an inactive one or one of greatly reduced activity due to premature chain termination. Accordingly, to isolate such NR-free mutants, a procedure was adopted in which the frame-shift mutagen, 9-aminoacridine, was the mutagen used to induce the NR-deficiency and NIR was the selecting agent (17). Indeed such NIR-resistant mutants (e.g., TA98/NIR-301) were much more resistant to the mutagenic action of NIR than colonies isolated in the conventional method, i.e., in the absence of 9-aminoacridine (e.g., TA98/NR) (17). This is consistent with the hypothesis that the strains isolated in the presence of 9-aminoacridine are frameshift mutants lacking in NR while the "conventionally" isolated strains are "leaky", their residual activity being due to the minimal activity of the amino acid-substituted enzyme. However, both the "conventional" as well as the frameshift mutants were fully responsive to the mutagenicity of 1,8-DNP (Table I) which is evidence in support of the hypothesis that these substrates are acted upon by different enzymes. Moreover it is plausible that the residual activity of NIR (0.2%) exhibited by the frameshift mutants (e.g., TA98/NIR-301) is due to reduction of this nitro-compound by an alternate NR (17). It should be noted that all of the tester strains were sensitive to the mutagenic action of NQO. Since reduction of the nitro group of NQO is essential for its mutagenicity, this provides added support for the existence of an additional NR (18).

If it be argued that the differences in substrate specificity of the various mutants (i.e., TA98NR and TA98/1,8-DNP) result from mutations affecting the active site of a single enzyme then it is predicted that double-mutants, i.e., resistant to both types of substrates, and therefore lacking both substrate-recognizing sites should not exist. To test this hypothesis TA98/NR was rendered resistant to 1,8-DNP (TA98/NR/1,8-DNP) and *vice versa* TA98/1,8-DNP$_6$ was made resistant to NIR (TA98/1,8-DNP/NR). All of the double mutants, irrespective of the order in which they were isolated, exhibited the expected phenotype, i.e., they were resistant to the mutagenicity of NIR as well as to that of 1,8-DNP (Table II). In addition they retained full sensitivity to the mutagenicity of NQO.

The data described above provide evidence that *Salmonella* contain at least two different gene products endowed with NR activity. Further studies will be needed to establish the structural existence of additional NRs that have been implicated. However, the investigations described herein encourage us to undertake additional experiments to determine the properties of these putative enzymes. It also remains to be ascertained whether this multiplicity of enzymes is unique to bacteria or whether it has a parallel in mammalian systems. An analysis of the environmental risks posed by nitroarenes is dependent upon such a determination (6,19). A finding which may have a bearing on this subject is the demonstration that nitropyrenes which are potent bacterial mutagens are devoid of recombinogenic activity in *Saccharomyces cerevisiae*, a species which may lack the appropriate NR yet which is responsive to the genetic activity of the nitrofuran, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide and to NQO (1,20). In addition it was found that microsomal preparations from mammalian livers contained potent enzymes which eliminated the mutagenicity of nitropyrenes for bacteria (1,9). The existence of a family of NRs in *Salmonella* is not implausible. The detection of a group of "classical" NRs in *E. coli* had been described earlier by McCalla and his associates (21,22). Thus, in addition to the oxygen-sensitive activity, three oxygen-insensitive nitrofuran reductase activities were identified (22).

Table I

Properties of *Salmonella typhimurium* TA98 strains deficient in classical nitroreductases[a]

Strain	NIR (Rev/μg)	Mutagenicity: % of TA98 NIR	1,8-DNP	NQO
TA98	1810	100	100	100
TA98/NR	80	4.4	104	100
TA98/NIR-301	3.5	0.2	106	100
/NIR-302	3.5	0.2	100	100
/NIR-303	2.9	0.2	100	100
/NIR-304	4.0	0.2	100	100

[a]A total of 19 NIR strains were isolated.
The results summarized above were calculated from the linear portion of the dose-response curves. The reproducibility of the results within each series of experiments was ± 4%.

Table II

Properties of derivatives of *Salmonella* TA98 deficient in two nitroreductases

Strain	Mutagenicity: % of control 1,8-DNP	NIR	NQO
TA98	100	100	100
TA98/NIR	104	4	100
TA98/DNP$_6$	2	100	84
TA98/NR/DNP$_1$	1.3	0	100
NR/DNP$_2$	1.4	0	100
NR/DNP$_5$	3.9	0	100
TA98/DNP$_6$/NR$_1$	2	0	100
DNP$_6$/NR$_8$	2	0	100
DNP$_6$/NR$_{11}$	2	0	100
DNP$_6$/NR$_{12}$	2	0	100

Mutagenic activities were calculated from the linear portion of the dose-response curves. These values were 257 000, 400 and 128 revertants/nmol for 1,8-DNP, NIR and NQO, respectively, in strain TA98.

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